Colchicine Antimitosis Abolishes Resiliency of Postnatally Developing Rats to Chlordecone-amplified Carbon Tetrachloride Hepatotoxicity and Lethality

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We have previously reported that rats are resilient to the hepatotoxic and lethal combination of chlordecone (CD) and carbon tetrachloride (CCl_4) during early postnatal development. The overall findings pointed to stimulated cell division and tissue repair mechanisms as the underlying cause of resistance. The objective of the current study was to investigate if the antimitotic effect of colchicine (CLC) abolishes this resiliency to CD + CCl_4 by inhibiting ongoing and stimulated cell division. We used 45-day-old rats in this study because this age group exhibited partial sensitivity to CD + CCl_4 in our previous studies. Male Sprague-Dawley rats were treated with a single low intraperitoneal dose of CCl_4 (100 µl/kg) or corn oil after exposure to either 10 ppm CD in the diet or a normal diet (ND) for 15 days. CLC (1 mg/kg) was administered 6 or 30 hr after CCl_4 to ND or CD rats, respectively. Administration of CLC resulted in increased CCl_4-induced mortality from 25% to 85% in rats pretreated with CD, in contrast to 100% survival in ND rats. Liver injury was assessed by plasma alanine transaminase (ALT) and sorbitol dehydrogenase (SDH) elevations as well as by histopathology. Hepatocellular regeneration was assessed by ^{3}H-thymidine (^{3}H-T) incorporation into hepatonuclear DNA and proliferating cell nuclear antigen (PCNA) studies during 0–96 hr after CCl_4. Administration of CLC to ND + CCl_4 rats resulted in a slight delay in cell division and tissue repair, as indicated by ^{3}H-T incorporation and PCNA, thereby leading to prolonged liver injury as revealed by elevations in plasma ALT, SDH, and histopathological lesions. In contrast, CLC administration to CD + CCl_4-treated rats further delayed and diminished cell division by 80%, which led to unrestrained progression of CCl_4-induced liver injury, resulting in 85% mortality. These findings underscore the importance of ongoing and toxicant-stimulated cell division and tissue repair mechanisms in hepatotoxicity, and the need for the inclusion of age factors in risk assessment of exposure to environmental and other chemicals. *Key words*: carbon tetrachloride, chlordecone, colchicine, postnatal resiliency, tissue repair. *Environ Health Perspect* 106:597-606 (1998). [Online 17 August 1998]
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Previous studies demonstrated that chemical-induced hepatic injury and disease can either increase or decrease with age (*1*). Recent studies have revealed that neonates and young rats are resilient to a wide variety of structurally and mechanistically dissimilar hepatotoxicants such as CCl_4 (*2–6*), allyl alcohol (*7*), galactosamine (*8*), and acetaminophen (*9*). Furthermore, young rats are resilient to the combination of chlordecone (CD) and CCl_4 (*4–6*), which is known to cause fulminant hepatic failure (*10*) and 100% mortality in adult rats (*10–12*).

Carbon tetrachloride is a hepatotoxic halomethane capable of causing hepatocellular fatty degeneration and centrilobular necrosis (*13,14*). The widely accepted mechanism of CCl_4-initiated liver injury involves the bioactivation of CCl_4 by the cytochrome P450-mediated reactions to the $^{\bullet}CCl_3$ free radical, which is further converted to a peroxy radical, $CCl_3O_2^{\bullet}$ (*15*). These free radicals readily react with polyunsaturated fatty acids to initiate lipid peroxidation. In the presence of cellular O_2, peroxy radicals in turn can react with other polyunsaturated fatty acids to perpetuate a series of self-propagating chain reactions that lead to runaway lipid peroxidation. It is well documented that stimulation of hepatocellular regeneration and tissue repair mechanisms play a critical role in recovery from the limited liver injury inflicted by a low dose of CCl_4 (100 µl/kg) (*16,17*). Prior exposure to a nontoxic dose of CD (10 ppm in diet for 15 days) is known to amplify hepatotoxicity and lead to lethality by a low dose CCl_4 (100 µl/kg) in adult rats (*10–12*). Studies have revealed that the CD + CCl_4 combination inhibits CCl_4-induced liver cell division and tissue repair in part through the depletion of hepatocellular energy, thereby permitting progression of injury leading to hepatic failure and animal death (*11,12,17–23*).

Earlier studies have suggested that newborns are less sensitive to chemicals like CCl_4 because of lower cytochrome P450 levels required for the bioactivation of CCl_4 to a toxic species (*2,3,9*). However, recent ^{3}H-T incorporation and proliferating cell nuclear antigen (PCNA) studies indicate that younger rats (45 days old) exhibit resiliency not only to CCl_4 alone, but also to the lethal combination of CD and CCl_4. This resiliency was closely associated with enhanced plasticity in hepatocellular regeneration and efficient tissue repair mechanisms in the younger animals (*4–6*), rather than due to differences in cytochrome P450 levels or bioactivation-dependent mechanisms (*4*). Furthermore, young rats are also resilient to hepatotoxicants such as galactosamine, which do not depend on cytochrome P450 bioactivation to inflict liver injury (*8*). These studies indicate that timely and efficient cell division are the key events in the resiliency of younger rats to hepatotoxicants.

The time-course studies (*4–6*) revealed that the loss of resiliency in adult rats may be due to insufficient cell proliferation and tissue repair, leading to unrestrained progression of injury rather than infliction of more severe injury. If cell division is responsible for the resiliency exhibited by postnatally developing rats, then inhibition of cell division by antimitotic agents (e.g., colchicine) should lead to a progression of CCl_4-induced hepatotoxicity and lethality. Therefore, the objective of the present study was to investigate if intervention with cell division and tissue repair by the antimitotic colchicine abolishes the resiliency of postnatally developing rats to CCl_4 and CD + CCl_4 hepatotoxicity and lethality. We report here that mitotic intervention significantly diminishes the resiliency of postnatally developing rats to CCl_4 toxicity. The findings of this study underscore the critical role of cell division and tissue repair in the final outcome of hepatic injury during early postnatal development. Most importantly,

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from a public health perspective (in particular child health), the results of this study may suggest consideration of a new paradigm in risk assessment and management of exposure to a mixture of environmental chemicals.

Materials and Methods

Animals. Postnatally developing (30-day-old) male Sprague-Dawley rats were obtained from our Central Animal Facilities and were maintained under controlled conditions of temperature (20°C ±1), relative humidity (50–80%), and illumination (12 hr light, 12 hr dark). All rats had free access to commercial powdered food (4% crude fat rat/mouse diet #7001, Teklad, Indianapolis, IN) and water. All animal husbandry and handling conditions were according to the Institutional Animal Care and Use Committee guidelines.

Treatment. Rats were maintained on the standard rodent powdered diet with (CD) or without (ND) 10 ppm CD for 15 days as described by Curtis and Mehendale (*19*). On day 16 (when the rats turned 45 days old), rats from each dietary protocol were challenged with a single intraperitoneal (ip) dose of CCl_4 (100 µl/kg) or corn oil. Blood and liver samples were collected at 0, 6, 12, 24, 36, 48, 72, and 96 hr after CCl_4 or corn oil administration. The blood samples were used to determine plasma alanine transaminase (ALT) and sorbitol dehydrogenase (SDH), whereas the liver samples were used to determine 3H-T incorporation into hepatic nuclear DNA (see below for pulse-labeling studies), for histopathology, and for PCNA analyses. Colchicine (CLC; 1 mg/kg) was administered in normal saline (NS) to ND and CD rats at 6 and 30 hr after CCl_4 administration, respectively. Colchicine was administered 6 hr before the maximum stimulation (12 hr for ND and 36 hr for CD) of DNA synthesis as assessed by ^{3}H-T incorporation studies in both ND + CCl_4 and CD + CCl_4 treatment groups. Similarly, blood and liver samples were obtained during a time-course of 0–96 hr for biochemical and pathological studies. Colchicine at 1 mg/kg has been shown to have no measurable effects on the metabolism, disposition of CCl_4, or function of the liver (*23*).

Assessment of liver injury. We measured plasma enzymes ALT (EC 2.6.1.2) and SDH (EC 1.1.1.14) as markers of liver injury according to the methods of Bergmeyer et al. (*24*) and Asada and Galambos (*25*), respectively. This analysis was corroborated by hepatopathology using 10% formalin-fixed, paraffin embedded, 5-µm thick sectioned tissues, stained with hematoxylin and eosin (H&E) (*26*).

Assessment of hepatocellular proliferation. We measured hepatic cell proliferation using ^{3}H-T incorporation into hepatonuclear DNA and PCNA techniques. ^{3}H-thymidine was administered at a dose of 50 µCi/300 g body weight (ip) 2 hr before sacrifice. The procedure used to isolate hepatonuclear DNA was that described by Chang and Looney (*27*). Briefly, 1 g of liver sample was homogenized in 20 w/v 2.2 M sucrose solution at 4°C. The homogenates were centrifuged at 40,000*g* for 1 hr in a Beckman L5-75 ultracentrifuge (Beckman Instruments, Palo Alto, CA). The sedimented nuclei were washed twice with 0.25 M sucrose. To 1 ml of nuclear suspension we added 0.5 ml of 0.6N perchloric acid (PCA) and centifuged at 10,000*g* for 20 min. The nuclear content was ultimately suspended in 6 ml of 0.5 N PCA and centrifuged at 10,000*g* for 20 min. Liver DNA content was measured by diphenylamine reaction as described by Burton (*28*). To 1 ml of extracted DNA solution, 2 ml of diphenylamine reagent was added and incubated at room temperature for 16–18 hr. DNA content was determined spectrophotometrically at 600 nm. Two aliquots of 200 µl of the DNA supernatants were added to 10 ml Scientiverse-E to quantify radioactivity using a Packard scintillation counter (Packard Instrument Company, Meriden, CT).

The PCNA study was conducted as described by Greenwell et al. (*29*). Briefly, formalin-fixed liver tissues were processed for paraffin embedding. Liver sections (5-µm thick) were mounted on poly-L-lysine-treated slides, deparaffinized, and subjected to 3% H_2O_2 to quench endogenous peroxide activity. Tissues were blocked with 0.5% casein to suppress nonspecific binding of IgG. Liver sections were then incubated with monoclonal primary antibody to PCNA (Dako Corp., Carpentaria, CA), linked with biotinylated goat anti-mouse IgG antibody (Boehringer/Mannheim, Indianapolis, IN), and labeled with streptavidin-conjugated

Table 1. Effects of colchicine antimitosis on lethality of CD + CCl_4 combination in 45-day-old rats[a]

Group	Treatment	*n*	% Lethality	% Survival
I	ND + CO + CLC	10	0	100
II	ND + CCl_4 + NS	10	0	100
III	ND + CCl_4 + CLC	10	0	100
IV	CD + CCl_4 + NS	20	25	75
V	CD + CCl_4 + CLC	20	85	15

[a]Male Sprague-Dawley rats were maintained on either normal diet (ND) or 10 ppm chlordecone (CD) in the diet for 15 days. On day 16, a non-toxic dose of carbon tetrachloride (CCl_4) (100 µl/kg, ip) was administered in corn oil (CO) to all rats. Rats maintained on ND and those maintained on CD received colchicine (CLC) at 6 hr prior to maximum S-phase stimulation induced by CCl_4 (100 µl/kg, ip). The animals were observed twice daily for 14 days. All deaths in CD + CCl_4 + NS-treated rats occurred between 36 and 72 hr, whereas in CD + CCl_4 + CLC-treated rats, it occurred between 36 and 96 hr after administration of CCl_4. NS, normal saline.

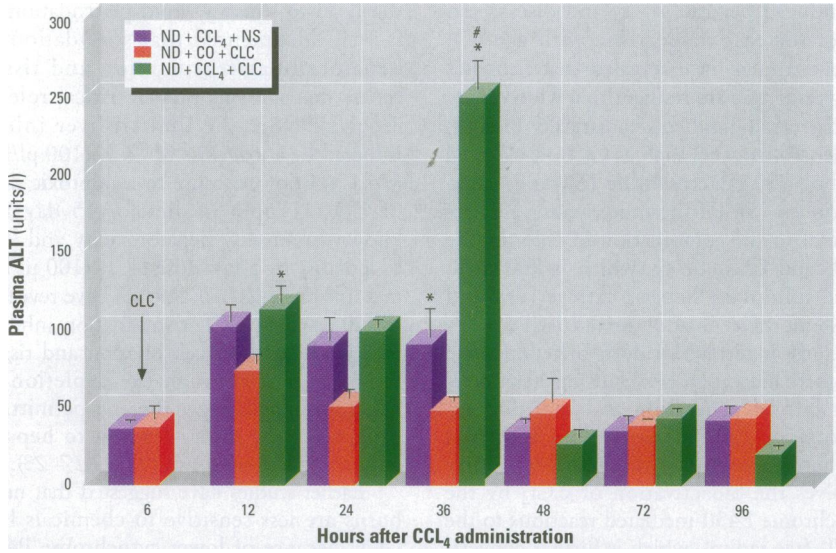

Figure 1. Effects of colchicine (CLC) antimitosis on plasma alanine transaminase (ALT) in rats maintained on normal diet (ND) with or without carbon tetrachloride (CCl_4) treatment. The rats received CLC (1 mg/kg, ip) 6 hr before S-phase stimulation after administration of CCl_4 (100 µl/kg, ip). Plasma ALT levels were monitored during a time-course after CCl_4 (100 µl/kg, ip) administration to 45-day-old rats maintained on ND. Values are expressed as means ± standard errors of four rats.
*Significant increase in ALT levels as compared to the respective control [ND + corn oil (CO) + CLC] of same time point.
#Significantly higher ALT levels compared to that in ND + CCl_4 + normal saline (NS) rats ($p \leq 0.05$).

peroxidase (Jackson Immunoresearch, West Grove, PA). Color was developed by 3,3′-diaminobenzidine, and cells were counterstained with hemotoxylin (Sigma, St. Louis, MO). Cells in various phases of the cycle were identified as follows: G_0, cells with no staining; G_1, cells with light brown nuclear staining; S, cells with deep brown nuclear staining; G_2, cells with diffuse speckled nuclear staining and brown cytoplasmic staining; and M, cells with diffuse cytoplasmic staining with deep blue chromosomal staining. Quantitation of the various cell cycles were conducted using Leica's computer image analyzer Q 500MC (Leica, Deerfield, IL).

Statistical analysis. The data are presented as mean ± standard error of the mean (SEM). We used two-way analysis of variance followed by pairwise comparisons of selected means using the pooled within-group variance comparisons. The criterion for significance was set at $p \leq 0.05$.

Results

Lethality study. The lethality study was conducted to examine if stimulated cell division is critical in the resiliency of postnatally developing rats to CD-potentiated CCl_4 hepatotoxicity and lethality. This concept was tested in 45-day-old rats using CLC antimitosis because this age group is known to exhibit partial sensitivity (25% mortality) to CD + CCl_4 (*4,5*). The administration of CLC (1 mg/kg, ip) to the ND + CCl_4 group did not result in any mortality (Table 1). In contrast, CLC increased the mortality from 25% to 85% in 45-day-old CD + CCl_4- treated rats.

Assessment of hepatic injury. Plasma ALT and SDH were measured in 45-day-old rats as markers of liver injury 12, 24, 36, 48, 72, and 96 hr after the administration of CCl_4. Treatment with CCl_4 alone resulted in transient and marginal liver injury as evidenced by ALT elevation at 12 hr (Fig. 1) and SDH elevation (not shown). Thereafter, liver injury declined, leading to complete recovery by 48 hr. Suppression of stimulated cell division by CLC administered 6 hr after CCl_4 treatment resulted in elevation of plasma ALT level as early as 12 hr, peaking at 36 hr (Fig. 1). Recovery from CCl_4-inflicted liver injury was evident by 48 hr after CCl_4. Thus, the limited and transient liver injury caused by a low dose of CCl_4 alone was increased in the presence of CLC treatment.

Pretreatment with CD dramatically amplified CCl_4-induced liver toxicity as evidenced by plasma enzyme levels. Administration of CCl_4 to CD-pretreated 45-day-old rats resulted in significant elevation of plasma ALT (Fig. 2) and SDH (data not shown) as early as 6 hr after the administration of CCl_4. Enzyme elevation progressed with time, with the peak occurring 36 hr after CCl_4. Injury was sustained until 48 hr, after which enzyme levels declined to control levels by 96 hr. The progression of liver injury was accompanied by 25% mortality, which occurred between 36 and 72 hr after CCl_4. Administration of CLC to the CD + CCl_4 group resulted in persistence of ALT elevations noted at 36 and 48 hr. In contrast to the CD + CCl_4 group, enzyme elevations were persistent even at 72 and 96 hr (Fig. 2).

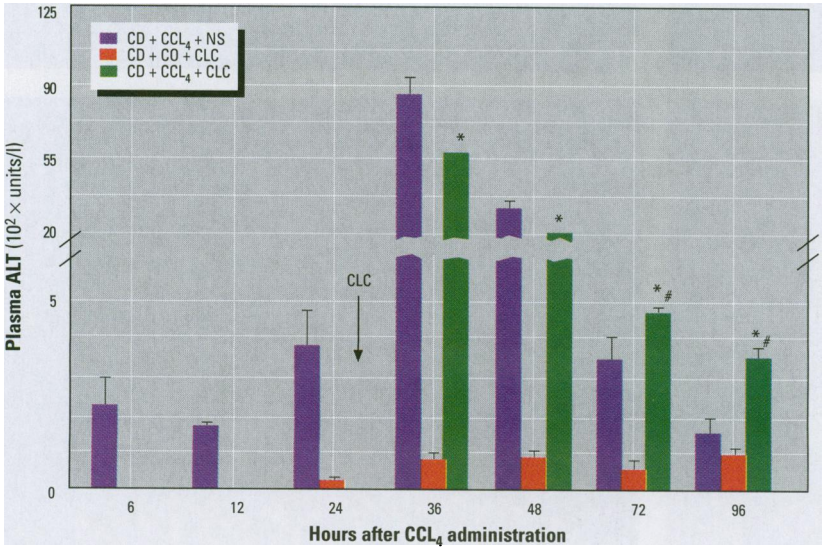

Figure 2. Effects of colchicine (CLC) antimitosis on plasma alanine transaminase (ALT) in rats maintained on 10 ppm chlordecone (CD) diet for 15 days. The rats received CLC (1 mg/kg, ip) 6 hr before S-phase stimulation induced by carbon tetrachloride (CCl_4; 100 µl/kg, ip). Plasma ALT levels were measured during a time-course after CCl_4 (100 µl/kg, ip) administration. Values are expressed as means ± standard errors of four rats.
*Significant increase in ALT levels as compared to the corresponding control [CD + corn oil (CO) + CLC] of the same time point.
#Significantly higher ALT levels compared to CD + CCl_4 + normal saline (NS) rats ($p \leq 0.05$).

This sustenance in injury was concomitant with increased mortality (85%, which occurred between 36 and 96 hr after CCl_4) noted in the CD + CCl_4 + CLC group, compared to the 25% mortality noted in the CD + CCl_4 + NS group.

Histopathological alterations were assessed in liver sections by light microscopy for necrotic, swollen (ballooned), and lipid-laden hepatocytes (Figs. 3 and 4). Histopathological changes were consistent with the plasma enzyme elevations. Irrespective of pretreatment, CCl_4-induced liver injury was evident around the centrilobular region as early as 6 hr after CCl_4 treatment. The injury progressed with time, as evidenced by the extent of pyknotic nuclei and swollen, lipid-laden hepatocytes. No histopathological alterations were evident in the livers of control rats (0 hr time point: Figs. 3A,B and 4A,B) regardless of dietary treatment.

In ND rats, CCl_4-induced liver injury was evident as early as 6 hr after CCl_4 administration, with the maximum injury at 12 hr (Fig. 3C), followed by recovery by 48 hr (Fig. 3E). Administration of CLC to ND + CCl_4-treated rats resulted in more severe and prolonged liver injury, although it did not affect recovery from CCl_4-induced liver injury (Fig. 3D). Similarly, CCl_4-induced liver injury in CD-pretreated rats was evident as early as 6 hr after CCl_4 administration. However, injury progressed with time, with maximum injury observed at 48 hr (Fig. 4C), until 25% mortality occurred within 72 hr. The morphological alterations in the livers of the surviving 75% of 45-day-old rats were minimal at 96 hr after the administration of CCl_4 (Fig. 4E). In contrast, liver injury in CD + CCl_4 + CLC rats was higher than in ND + CCl_4 + CLC- or CD + CCl_4 + NS-treated groups (Fig. 4D). Furthermore, the rate of recovery from liver injury in CD + CCl_4 + CLC-treated rats (15% survivors) was delayed (Fig. 4F) compared to that seen in the 75% survivors of CD + CCl_4 + NS treatment group (Fig. 4E), with incomplete recovery even at 96 hr after CCl_4 administration.

Effects of colchicine on carbon tetrachloride-induced cell division. We used CLC to examine whether intervention of mitosis in the CD + CCl_4 rats decreased cell division and whether this correlated with increased mortality. In ND + CCl_4 rats, significant increases in ^{3}H-T incorporation occurred between 12 and 36 hr after the administration of CCl_4 (Fig. 5). However, administration of CLC resulted in blockage of S-phase stimulation at 12 and 24 hr, the maximum ^{3}H-T incorporation being shifted from 12–24 hr to 36 hr after the administration of CCl_4 (Fig. 5). This delay in cell division in ND + CCl_4 +

CLC-treated rats correlated with increased liver injury (Fig. 1).

In sharp contrast, the CD + CCl_4 + NS treatment resulted in significantly delayed S-phase stimulation as evidenced by the ^{3}H-T incorporation (Fig. 6). Consequently, maximum S-phase stimulation occurred at 72 hr compared to that occurring between 12 and 36 hr in the ND + CCl_4 + NS group (Fig. 5). The 75% survival in the CD + CCl_4 + NS treatment group is concordant with this substantial increase in tissue repair that occurred between 36 and 72 hr after CCl_4. Administration of CLC to the CD + CCl_4 group resulted in a marked suppression of S-phase synthesis (Fig. 6). Administration of CLC resulted in an 80% decrease in the maximum ^{3}H-T

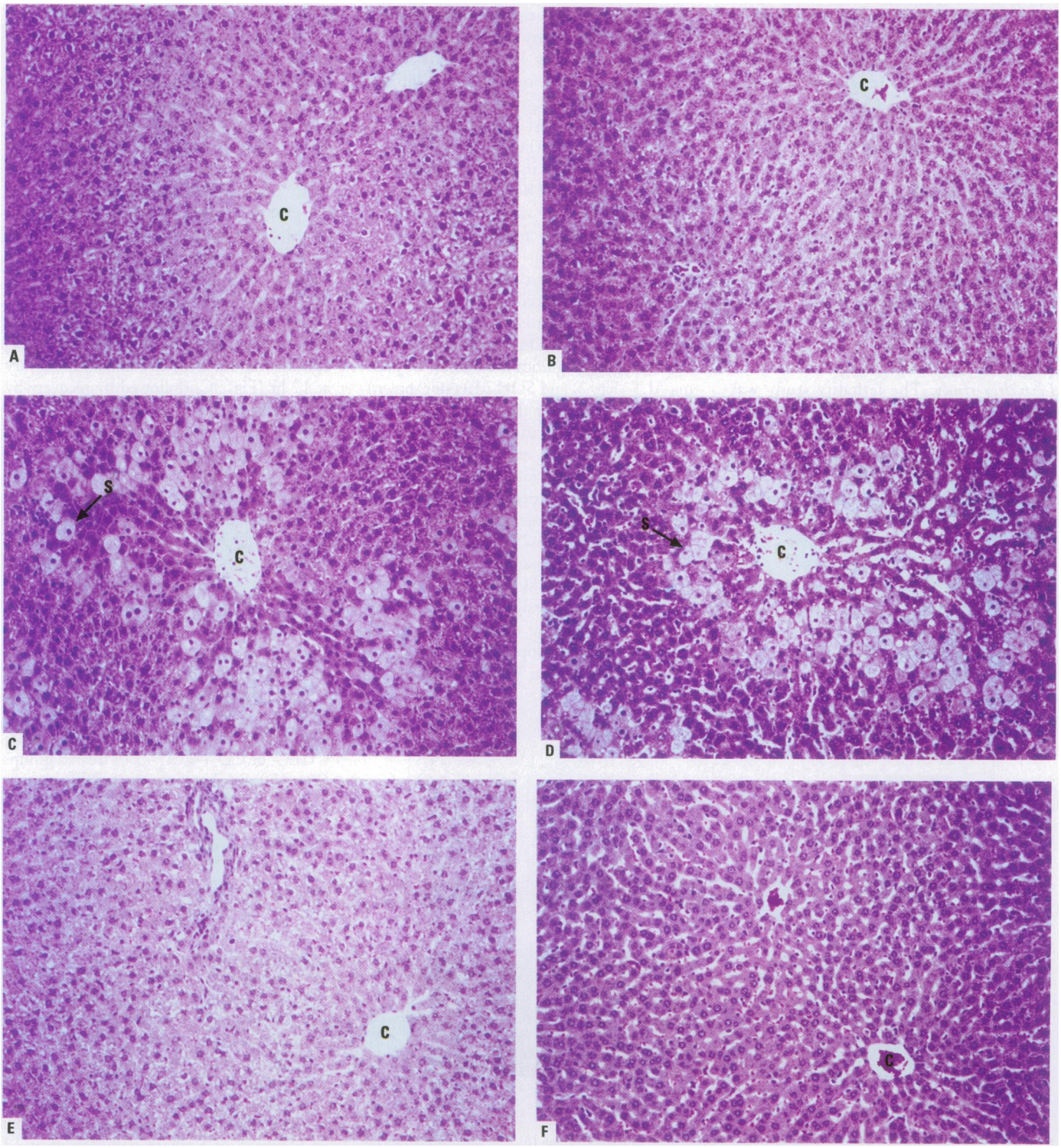

Figure 3. Representative photomicrograph of typical H&E-stained liver section from 45-day-old rats challenged with a single dose of carbon tetrachloride (CCl_4; 100 µl/kg, ip). (A) Normal diet (ND) + CCl_4 + normal saline (NS) at zero time point. (B) ND + CCl_4 + colchicine (CLC) at zero time point. (C) Time of maximum injury (12 hr after CCl_4) in the ND + CCl_4 + NS group. (D) Time of maximum injury (48 hr after CCl_4) in the ND + CCl_4 + CLC group. (E) Time of recovery (48 hr after CCl_4) in the ND + CCl_4 + NS group. (F) Time of recovery (48 hr after CCl_4) in the ND + CCl_4 + CLC group. ×112. Abbreviations: C, central vein; S, swollen and vacuolated cells.

incorporation in CD + CCl_4-treated rats at 72 hr after CCl_4 (Fig. 6).

The PCNA immunohistochemical staining was conducted to corroborate the findings in the ^{3}H-T incorporation study. This technique enables one to identify cells in various phases of the cell cycle. The PCNA study demonstrated a similar hepatocellular proliferative activity pattern as observed in the ^{3}H-T incorporation study. In control rats (zero time), almost all the cells were in the resting phase (G_0) of the cell cycle (Figs. 7A,B and 8A,B). Only about 3% of the total hepatocytes were in S-phase (Tables 2 and 3).

A significant number of cells progressed to G_1 and S-phase of the cell cycle by 12 hr after CCl_4 in ND + CCl_4 + NS rats. A

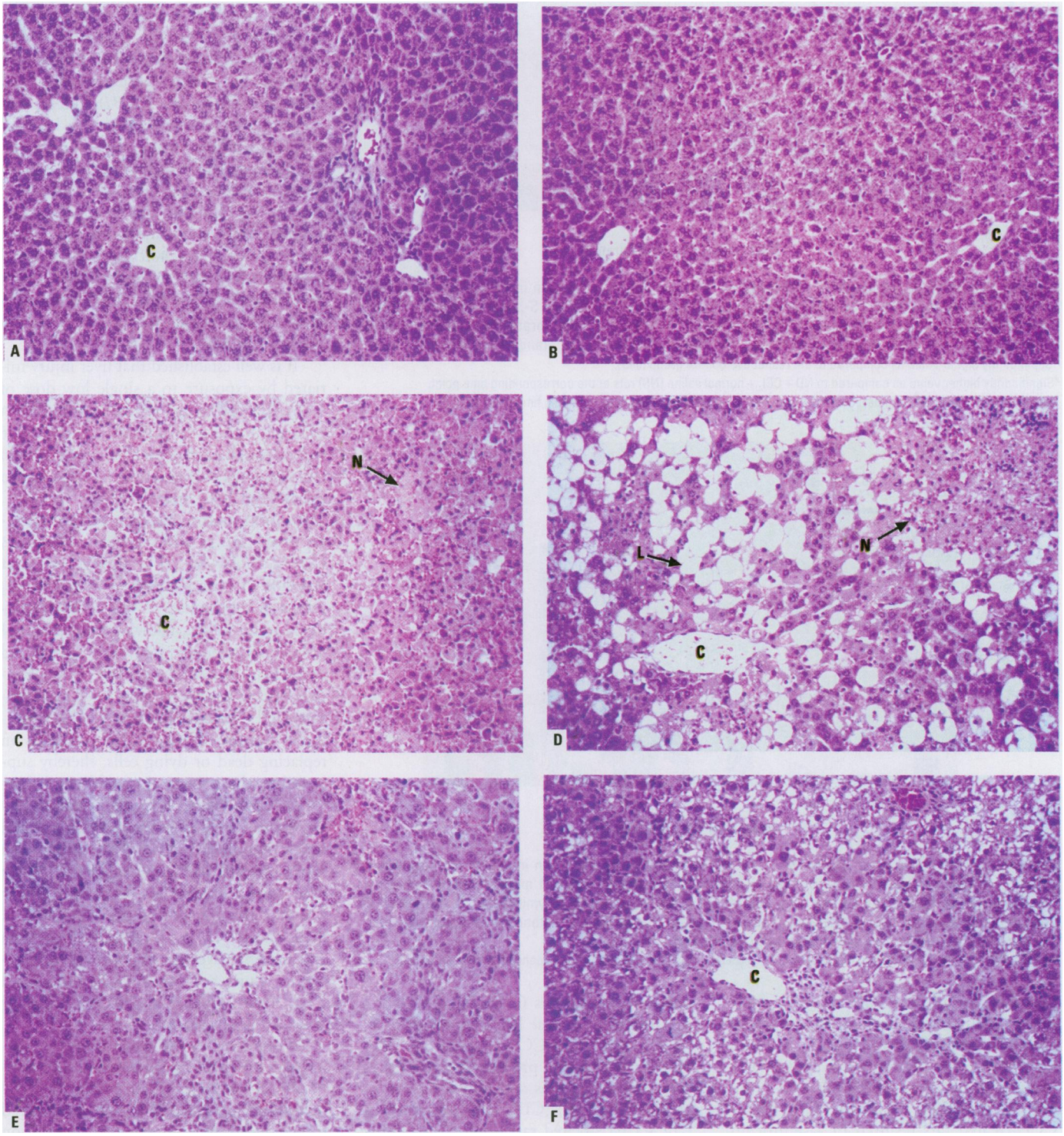

Figure 4. Representative photomicrograph of typical H&E-stained liver section from 45-day-old rats maintained on 10 ppm chlordecone (CD) diet for 15 days. On day 16, they were challenged with a single dose of carbon tetrachloride (CCl_4; 100 µl/kg, ip). (A) CD + CCl_4 + normal saline (NS) at zero time point. (B) CD + CCl_4 + colchicine (CLC) at zero time point. (C) Time of maximum injury (48 hr after CCl_4) in the CD + CCl_4 + NS group. (D) Time of maximum injury (72 hr after CCl_4) in the CD + CCl_4 + CLC group. (E) Time of recovery (96 hr after CCl_4) in the CD + CCl_4 + NS-treated surviving rats (75%). (F) Time of partial recovery (96 hr after CCl_4) in the CD + CCl_4 + CLC-treated surviving rats (15%). ×112. Abbreviations: C, central vein; N, necrosis; L, lipid-laden cells.

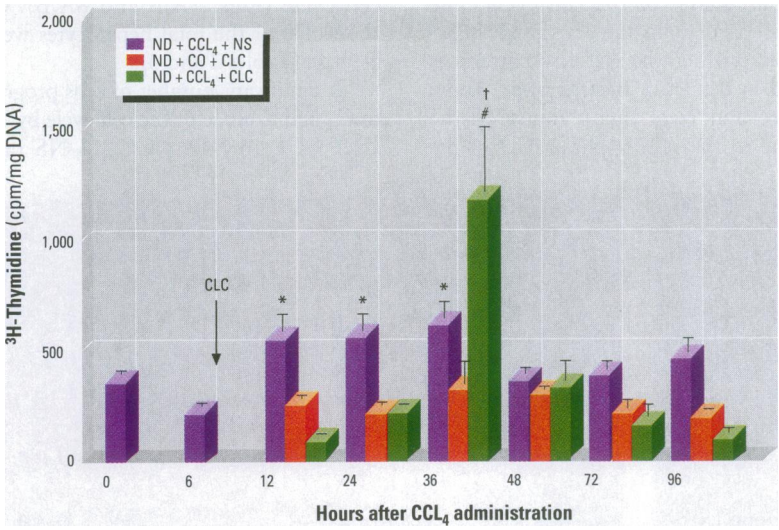

Figure 5. Effects of colchicine (CLC) antimitosis on ^{3}H-thymidine incorporation in 45-day-old rats maintained on normal diet (ND). The rats received CLC (1 mg/kg, ip) 6 hr before S-phase stimulation after administration of carbon tetrachloride (CCl_4; 100 µl/kg, ip). ^{3}H-Thymidine incorporation was measured during a time-course of 0–96 hr after CCl_4. Values are expressed as means ± standard errors of four rats.
*Significantly higher value as compared to the respective control group (0 hr).
#Significantly higher value as compared to ND + CCl_4 + normal saline (NS) rats at the corresponding time point.
†Significantly higher value compared to ND + corn oil (CO) + CLC rats at the corresponding time point. ($p \leq 0.05$).

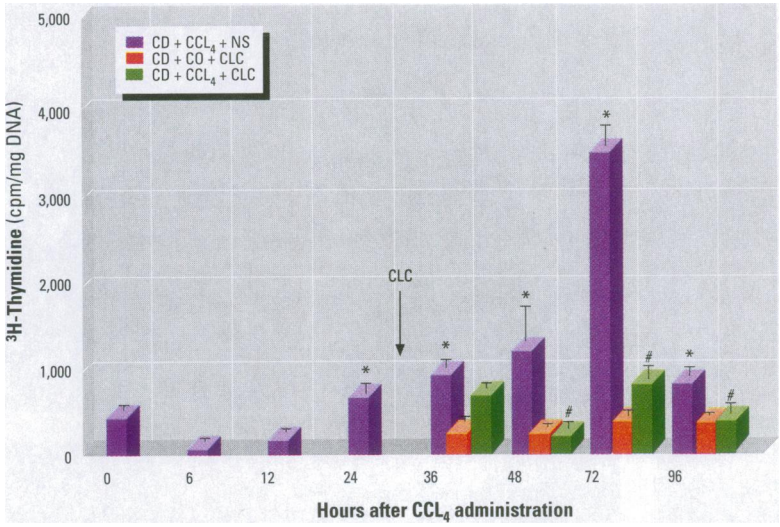

Figure 6. Effects of colchicine (CLC) antimitosis on ^{3}H-thymidine incorporation in 45-day-old rats maintained on 10 ppm chlordecone (CD) diet for 15 days. The rats received CLC (1 mg/kg, ip) 6 hr before S-phase stimulation induced by carbon tetrachloride (CCl_4; 100 µl/kg, ip). ^{3}H-Thymidine incorporation was measured during a time-course after CCl_4. Values are expressed as means ± standard errors of four rats.
*Significantly higher value as compared to their control group (0 hr).
#Significantly lower value as compared to CD + CCl_4 + normal saline (NS) rats at the corresponding time point ($p \leq 0.05$).

maximum number of cells were seen in S-phase (about 20%) between 24 and 36 hr after CCl_4 (Table 2 and Fig. 7C). These results are consistent with peak ^{3}H-T incorporation during the same time point (Fig. 5). Administration of CLC to ND + CCl_4-treated rats resulted in a significant reduction of cells in S-phase (from 20% to about 2%) 24 hr after the administration of CCl_4 (Table 2 and Fig. 7D). In contrast, in CD-pretreated rats, a significant increase in G_1 (10.3%) and S-phase (7.2%) cells occurred as early as 6 hr after CCl_4, the maximum in S-phase being noted at 72 hr (about 70%) after CCl_4 (Table 3 and Fig. 8C). By 96 hr, the proliferative activity of the hepatocytes was still above the background (Table 3). CLC administration to CD + CCl_4-treated rats resulted in significant reduction of cells in S-phase from 68% to 21% at 72 hr, a reduction by about 47%, compared to those treated with CD + CCl_4 + NS (Table 3 and Fig. 8D). Cell division (M-phase) needed for tissue repair was reduced in the CD + CCl_4 + CLC group during the same time point (Table 3). These findings are concordant with the ^{3}H-T pulse-labeling studies.

Discussion

In infants and children exposed to environmental toxicants, risk may differ qualitatively and quantitatively from adults for a number of reasons, including physiology, metabolism, pharmacokinetics, diet, and physical environment (*30,31*). Infants and children may be more sensitive or less sensitive depending on the target organ and the chemicals to which they are exposed. Moreover, because these processes can change rapidly and can counteract one another, there is no simple way to predict the kinetics and sensitivity to chemical compounds in infants and children from data derived entirely from adult humans or from toxicity testing in adults and young animals (*32,33*).

It is well established that liver injury initiated by exposure to a single low dose of CCl_4 (100 µl/kg, ip) results in stimulation of hepatocellular proliferation and tissue repair mechanisms, which occur as an endogenous compensation in response to the limited liver injury inflicted by CCl_4. This compensatory response permits recovery from injury and survival of animals (*11,12,17,34–36*). It is also known that exposure to a combination of CD and CCl_4 results in suppression of this compensatory tissue repair in rats, allowing progression of liver injury, culminating in liver failure and animal death (*10–12,17,34–36*). In contrast, young rats are resilient to CD-potentiated CCl_4 hepatotoxicity and lethality primarily because of ongoing liver cell division replacing dead or dying cells, thereby suppressing the progression of liver injury (*4–6*). Furthermore, liver injury inflicted by a low dose of CCl_4 results in additional and timely stimulation of cell division over and above ongoing liver growth (*4–6*). This in turn allows restoration of hepatolobular structure and function in the liver. Both of these events (CCl_4-stimulated cell division and ongoing liver growth) provide protection to younger rats against CD-potentiated CCl_4 hepatotoxicity and lethality during postnatal development.

If cell division and tissue repair are critical determinants of the resiliency of postnatally developing rats, then blocking cell division by antimitotic agents should lead to prolonged and unrestrained progression of liver injury, ultimately leading to increased mortality. Forty-five-day-old rats experienced 25% lethality upon exposure to CD + CCl_4. If stimulated cell division and tissue repair are critical events for the

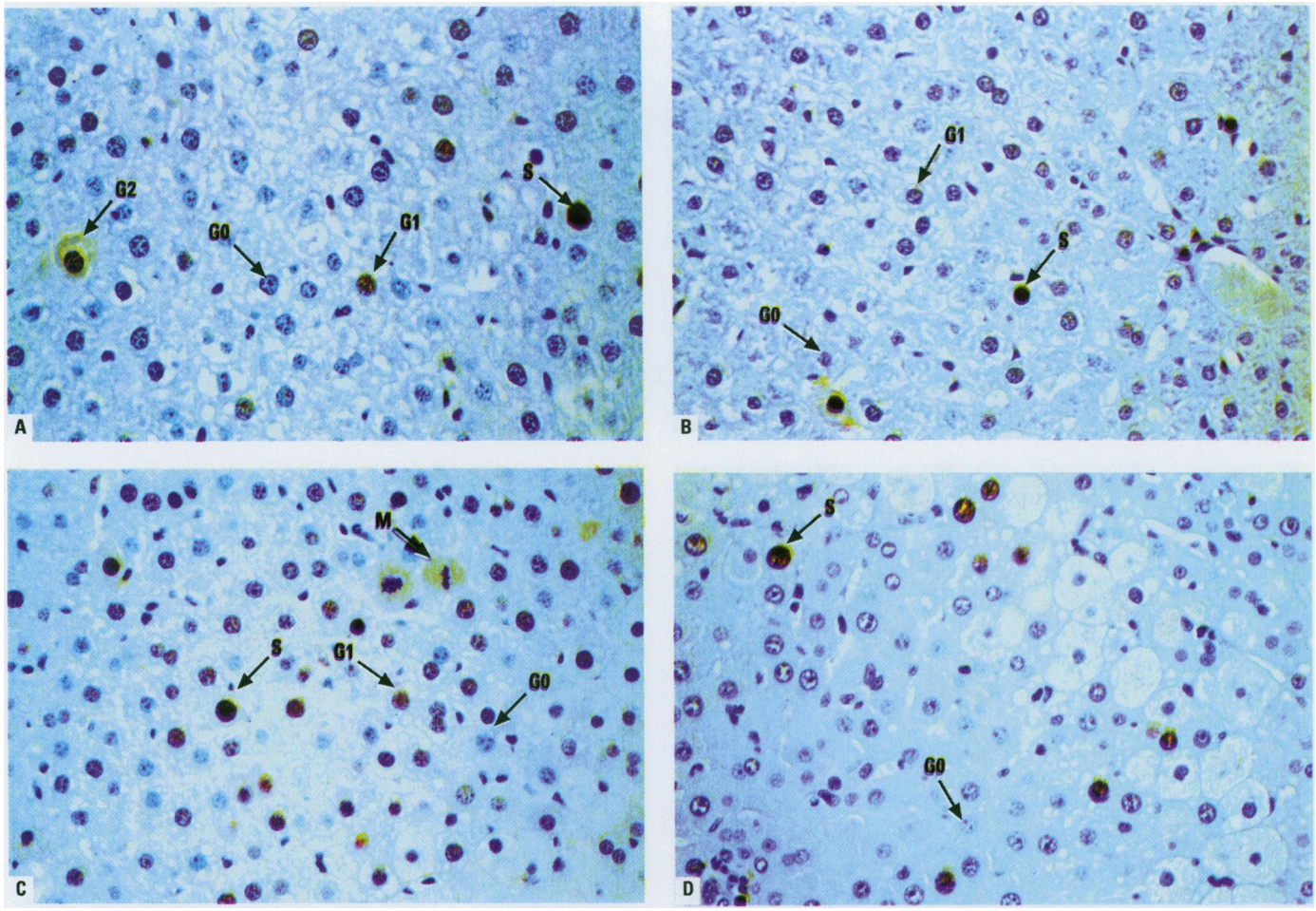

Figure 7. Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) during a time-course after carbon tetrachloride (CCl_4; 100 µl/kg, ip) administration to 45-day-old rats maintained on normal (ND) diet. A group of rats [ND + CCl_4 + colchicine (CLC)] received CLC (1 mg/kg, ip) before S-phase stimulation induced by CCl_4 (100 µl/kg, ip). (A) ND + CCl_4 + normal saline (NS) at zero time point. (B) ND + CCl_4 + CLC at zero time point. (C, D) Time at which maximum number of cells were seen in S, G_2, and M phases of the cell cycle for ND + CCl_4 + NS (24 hr after CCl_4) and ND + CCl_4 + CLC (36 hr after CCl_4) groups, respectively. (For details see Table 2.) G_0, cells with no staining; G_1, cells with light brown nuclear staining; S, cells with deep brown nuclear staining; G_2, cells with or without speckled nuclear staining and brown cytoplasmic staining; and M, cells with diffused cytoplasmic staining and with deep blue chromosomal staining. ×281.

resiliency of 75% of the surviving rats, intervening with cell division should lead to higher lethality in this group. Colchicine is an antimitotic agent often used in manipulation of cell division (*23,37,38*). Tsukamoto and Kojo (*39*) reported that CLC inhibits S-phase DNA synthesis by inhibiting thymidine kinase and thymidylate synthetase enzymes responsible for DNA synthesis. Colchicine is also known to disrupt cell division by preventing proper assembly of microtubules in the mitotic spindle (*40*). Colchicine at a dose of 1 mg/kg is an effective antimitotic agent with no measurable side effect (*37,38*) or any influence on the metabolism and disposition of CCl_4 (*23*). In 45-day-old rats, treatment with CCl_4 alone resulted in limited liver injury, which goes through the progressive phase up to 12 hr with subsequent recovery via stimulation of tissue repair, leading to restoration of the damaged tissue with no lethal consequence. Colchicine antimitosis in this group (administered 6 hr after CCl_4) results in selective inhibition of the early phase of hepatic cell division after a low dose of CCl_4 (*23*). Because of inhibited early phase stimulation of cell division, the limited liver injury inflicted by CCl_4 is increased, as evidenced by plasma ALT (Fig. 1) and SDH elevations. However, significant cell division that occurs at later time points leads to complete recovery from liver injury.

Colchicine intervention in CD + CCl_4 rats resulted in even more accelerated progression of liver injury, which led to significantly higher lethality. Colchicine blocked DNA synthesis and cell division, leading to diminished and delayed hepatocellular regeneration and tissue repair in CD + CCl_4-treated rats. This was evidenced by 80% reduction in ^{3}H-T incorporation compared to CD + CCl_4 + NS-treated rats (Fig. 6). A similar pattern can also be seen at 72 hr after CCl_4 with PCNA studies, where CLC treatment resulted in about 47% reduction of cells in S-phase of cell cycle compared to those treated with CD + CCl_4 + NS (Table 3). Consequently, liver injury progressed in an unrestrained manner. Persistence of liver injury coupled with insufficient tissue repair led to hepatic failure in a majority of the 45-day-old rats receiving CD + CCl_4 + CLC treatment. This resulted in increased lethality from 25% to 85% (Table 1), with all deaths occurring by 96 hr after the administration of CCl_4. Highly accelerated progression of liver injury upon CLC administration is evident from persistent plasma enzyme levels and is corroborated by histopathology. The survival of 15% of the rats is presumably due to a much higher tissue repair, as indicated by higher DNA synthesis at 72 and 96 hr (Table 3, Figs. 6 and 8). Another study by Rao et al. (*41*), in which dose–response relationship between liver injury and compensatory tissue repair were

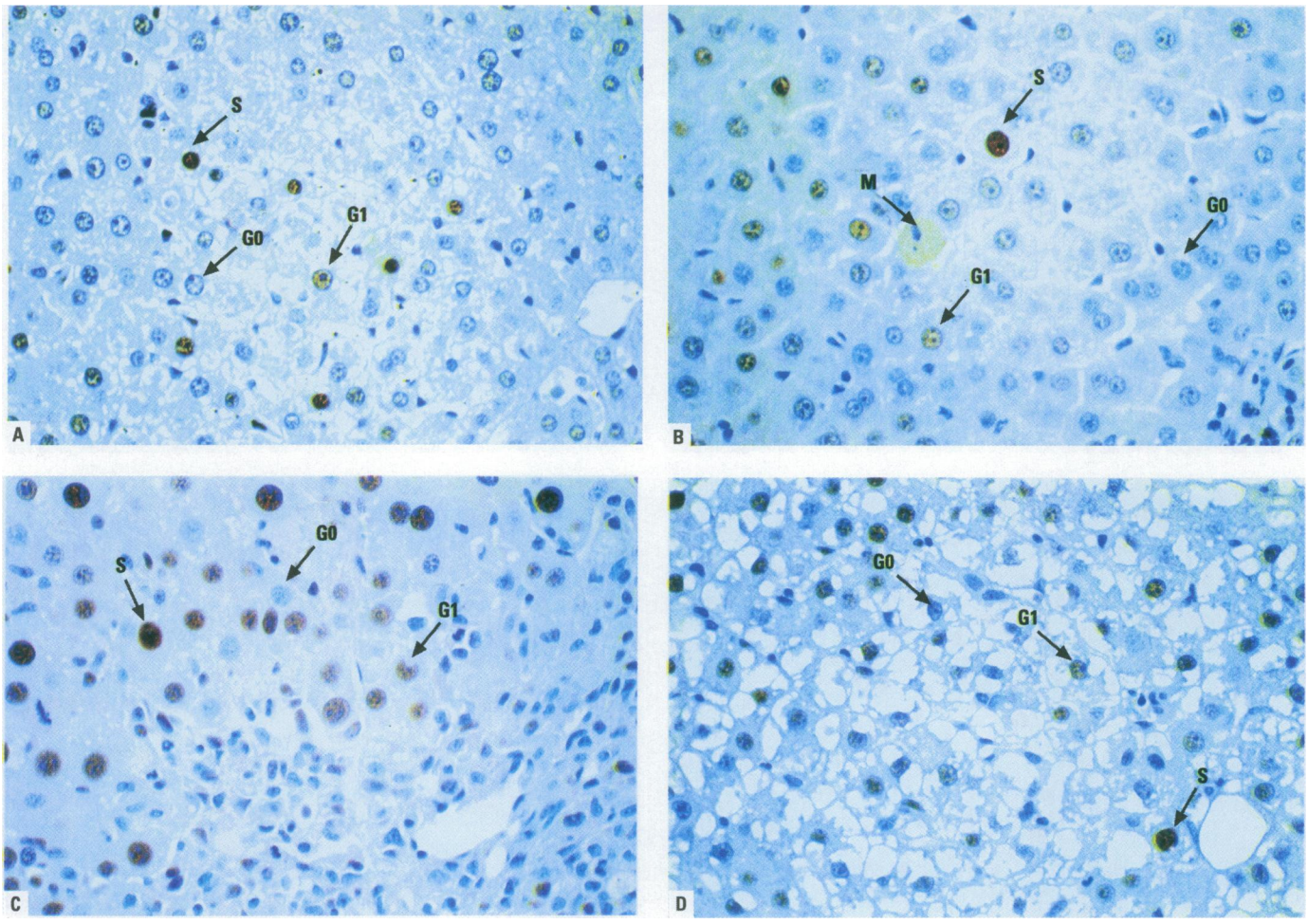

Figure 8. Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) during a time-course after carbon tetrachloride (CCl_4; 100 µl/kg, ip) administration to 45-day-old rats maintained on 10 ppm chlordecone (CD) diet for 15 days. A group of rats [CD + CCl_4 + colchicine (CLC)] received CLC (1 mg/kg, ip) before S-phase stimulation induced by CCl_4 (100 µl/kg, ip). (A) CD + CCl_4 + normal saline (NS) at zero time point. (B) CD + CCl_4 + CLC at zero time point. (C, D) Time at which maximum number of cells were seen in S, G_2, and M phase for CD + CCl_4 + NS and CD + CCl_4 + CLC, respectively (72 hr after CCl_4 for both groups). (For details see Table 3.) G_0, cells with no staining; G_1, cells with light brown nuclear staining; S, cells with deep brown nuclear staining; G_2, cells with or without speckled nuclear staining and brown cytoplasmic staining; and M, cells with diffused cytoplasmic staining and with deep blue chromosomal staining. ×281.

examined in CCl_4-treated adult male rats, supports the critical role of cell division and tissue repair in animal survival. In this study, administration of a high dose of CCl_4 (4 ml/kg) consistently resulted in 20% survival. Examination of S-phase stimulation (^{3}H-T incorporation) and cell cycle progression (PCNA) revealed a five-fold increase in DNA synthesis in this small population of the survivors. This massive increase in DNA synthesis appeared to facilitate efficient tissue repair processes, enabling 20% survival of rats. This was presumably due to genetic variation in these outbred rats. Therefore, the resiliency to the lethal combination of CD + CCl_4 + CLC in a small number of rats is to be anticipated.

The overall results of this study indicate that 45-day-old rats maintained on ND fully recover from liver injury inflicted by CCl_4 (100 µl/kg) owing to combined compensatory effects of ongoing cell division, additional stimulation of hepatocellular regeneration, and restoration of hepatolobular architecture. Administration of CLC to the ND + CCl_4 group results in an increase in the limited injury. This transient increase in injury does not seem to affect recovery rate of the rats from CCl_4-inflicted liver injury. Treatment with CLC alone resulted in a slight increase in the ALT value at 12 hr (Fig. 1). A similar pattern of ALT values were reported by Rao and Mehendale (*23*). The slight increase in ALT values between 12 and 24 hr in rats receiving CLC did not translate to any noticeable liver necrosis. The same dose of CCl_4 in CD-treated 45-day-old rats leads to some decrease in compensatory response, resulting in 25% mortality. However, 75% of 45-day-old rats escape death from this lethal combination of CD and CCl_4 owing to ongoing liver growth and additional stimulation of cell division and tissue repair mechanisms. Mitotic intervention with CLC further decreased the survival in CD + CCl_4-treated rats from 75% to 15% (Table 1), which demonstrates the critical role of ongoing liver growth and stimulation of hepatocellular regeneration in the resiliency of postnatally developing rats to the lethal combination of CD and CCl_4.

Toxicity resulting from exposure to two or more chemicals at individually nontoxic doses is of great interest from the public health viewpoint because this exposure scenario is most common (*35,36,42,43*). The current EPA risk assessment procedures use a safety factor of 10 to account for population variability. Infants and children are not routinely included in risk assessment, and most environmental regulations are based on exposure data of adult males (*33,44*). These concerns led the U.S. Congress in 1988 and recent years to request the National Academy of Sciences/National Research Council to carefully examine this issue (*32*). A better understanding of why infants and children respond differently to

Table 2. Hepatocytes in different phases of cell cycle during a time course after CCl_4 in rats treated with ND + CCl_4 + NS or ND + CCl_4 + CLC

Hours after CCl_4	Percentage of cells in different phases of cell cycle[a]				
	G_0	G_1	S	G_2	M
ND + CCl_4 + NS					
0	86.1 (1.55)	9.4 (1.08)	3.5 (0.75)	0.7 (0.18)	0.4 (0.11)
6	70.9 (1.1)*	17.9 (0.7)*	8.5 (0.29)*	2.3 (0.25)*	0.6 (0.12)*
12	57.2 (3.84)*	22.1 (1.29)*	16.7 (0.94)*	3.2 (0.21)*	0.8 (0.07)*
24	55.5 (2.05)*	23.9 (2.34)*	19.9 (0.15)*	0.6 (.035)	0.2 (0.10)*
36	73.8 (5.27)*	10.2 (1.18)*	15.7 (1.20)*	0.2 (0.09)*	0.2 (0.05)*
48	92.3 (0.52)*	3.4 (0.33)*	4.2 (1.27)	0.1 (0.06)*	0.1 (0.04)*
72	76.8 (2.66)*	20.5 (1.44)*	2.6 (1.18)	0.2 (0.03)*	0.04 (0.04)*
96	79.1 (6.45)	13.6 (3.72)	5.6 (0.85)*	0.0 (0.00)*	1.8 (0.14)*
ND + CCl_4 + CLC					
12	88.1 (5.82)#	5.9 (0.46)*#	5.1 (0.22)*#	0.4 (0.09)*#	0.6 (0.31)*
24	90.9 (4.10)#	7.6 (0.70)*#	1.6 (0.69)*#	0.0 (0.00)*#	0.0 (0.00)*#
36	71.1 (2.24)*	15.3 (0.8)*#	9.3 (1.56)*#	1.7 (0.07)*#	2.6 (0.21)*#
48	93.9 (5.99)*	3.8 (1.37)*	2.2 (1.74)#	0.0 (0.00)*#	0.1 (0.04)*
72	89.7 (1.99)#	5.6 (1.02)*#	3.6 (0.76)	0.6 (0.16)*#	0.6 (0.10)*#
96	93.9 (3.09)#	4.6 (0.22)*#	1.4 (0.12)*#	0.0 (0.00)*	0.04 (0.03)*#

Abbreviations: CCl_4, carbon tetrachloride; ND, normal diet; NS, normal saline; CLC, colchicine.

[a]Percentage was calculated from 1,000 cells counted from five randomly selected fields per slide. Forty-five-day-old male Sprague-Dawley rats received a single nontoxic dose of CCl_4 (100 µl/kg, ip) in corn oil (n = 4). In a second group, CLC (1 mg/kg, ip in NS) was injected 6 hr after CCl_4. Values are means ± SE (in parentheses). Controls for each time point were not significantly different from zero time.

*Significantly different from control zero time ($p \leq 0.05$).

#Significantly different from the ND + CCl_4 + NS group of the same time point ($p \leq 0.05$).

Table 3. Hepatocytes in different phases of cell cycle during a time course after CCl_4 in rats treated with CD + CCl_4 + NS or CD + CCl_4 + CLC

Hours after CCl_4	Percentage of cells in different phases of cell cycle[a]				
	G_0	G_1	S	G_2	M
CD + CCl_4 + NS					
0	93.4 (2.55)	3.7 (0.95)	2.8 (0.12)	0.1 (0.05)	0.0 (0.00)
6	80.2 (2.32)*	10.3 (0.37)*	7.2 (1.58)*	2.1 (0.33)*	0.1 (0.04)*
12	79.2 (1.05)*	20.8 (2.07)*	10.5 (3.94)*	0.1 (0.05)	0.0 (0.00)
24	63.2 (3.13)*	16.6 (2.03)*	19.9 (4.46)*	0.1 (0.00)	0.02 (0.01)*
36	71.1 (4.67)*	11.5 (0.81)*	17.4 (1.07)*	0.0 (0.00)*	0.0 (0.00)
48	54.6 (2.52)*	8.4 (1.17)*	37.1 (2.53)*	0.0 (0.00)*	0.0 (0.00)
72	12.3 (3.01)*	4.5 (0.80)	67.6 (5.09)*	11.3 (1.46)*	4.4 (0.20)*
96	19.5 (1.47)*	16.4 (3.29)*	49.3 (2.57)*	8.7 (0.46)*	6.1 (0.32)*
CD + CCl_4 + CLC					
36	86.6 (1.90)*#	8.9 (3.94)*	4.4 (1.39)*#	0.0 (0.00)*	0.0 (0.00)
48	71.3 (0.35)*#	19.4 (0.88)*#	6.8 (0.83)*#	1.5 (0.44)*#	0.9 (0.17)*#
72	35.5 (2.80)*#	33.9 (1.95)*#	21.1 (0.56)*#	2.1 (0.06)*#	1.3 (0.18)*#
96	42.0 (5.23)*#	39.9 (4.51)*#	15.9 (3.45)*#	1.3 (0.07)*#	0.9 (0.08)*#

Abbreviations: CCl_4, carbon tetrachloride; ND, normal diet; NS, normal saline; CLC, colchicine; CD, chlordecone.

[a]Percentage was calculated from 1000 cells counted from five randomly selected fields per slide (n = 4). Forty-five-day-old male Sprague-Dawley rats were challenged with low dose of CCl_4 (100 µl/kg, ip) in corn oil following dietary CD (10 ppm) for 15 days. In a second group, CLC (1 mg/kg, ip in normal saline) was administered 30 hr after CCl_4. Values are means ± SE (in parentheses). Controls for each time point were not significantly different from zero time.

*Significantly different from control zero time ($p \leq 0.05$).

#Significantly different from the CD + CCl_4 + NS group of the same time point ($p \leq 0.05$).

environmental chemicals is clearly needed. The findings of this study shed some light on the importance of the tissue repair factor in the resiliency of younger rats to hepatotoxicants. Furthermore, these findings support the inclusion of age factors in risk assessment of pesticides and other environmental chemicals. In the context of liver injury from toxic chemicals, our present findings suggest that liver injury is initiated by well-established mechanisms. Liver injury is restrained from progression due to greater stimulation of tissue repair in the young rats. Consequently, younger rats are able to overcome liver injury and restore liver structure and function. In the livers of younger animals, compensatory tissue repair response to injury is prompt, presumably because of facilitatory humoral factors and more efficient tissue repair due to shorter time for cell cycle completion (*6*). The molecular mechanisms underlying this expedient and efficient tissue repair at younger ages are worthy of investigation.

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